J Dent Sci 2009;4(3):136–148

ORIGINAL ARTICLE

Reactions of connective tissue to self-etching/priming dentin bonding systems: oxidative stress, tumor necrosis factor α expression, and tissue reactions

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Received: May 8, 2009
Accepted: Jul 30, 2009

KEY WORDS:
biocompatibility;
cytokines;
dentin bonding agents;
immune response;
soft tissue

Background/purpose: Few data have been published concerning tissue and systemic responses to resinous dental materials. The aim of this study was to compare and evaluate the biocompatibility of four kinds of dental self-etching/priming adhesives by measuring tissue responses, local and systemic tumor necrosis factor (TNF) α expression, and oxidative stress parameters.
Materials and methods: Eighty rats were equally divided into 10 groups. Four dental adhesives (Clearfil SE Bond, iBond, Clearfil Protect Bond, and Adper Prompt L-Pop) were applied to connective tissue of the rats. In the control group, rats were operated on with no material being applied. Biocompatibilities of the bonding agents were evaluated according to tissue responses, histopathologic and biochemical TNF-α expressions, and levels of malondialdehyde, glutathione, superoxide dismutase and glutathione peroxidase activities 1 week and 1 month after initiation of treatment.
Results: All neutrophil levels and edema formation between the iBond group and the other groups were statistically significant after 1 week. Fibroblast levels in the Clearfil SE Bond group were higher than all other groups. Vascularization levels statistically differed between the Clearfil SE Bond and iBond groups, and between the Adper Prompt L-Pop and control groups. Tissue TNF-α levels statistically differed in all groups other than the control group. At the end of 1 month, the neutrophil level in the iBond group was higher than that in the control group. The differences in fibroblast levels after 1 month were statistically significant between the Clearfil SE Bond and Clearfil Protect Bond groups, and between the control and iBond groups. Tissue TNF-α levels were higher in the iBond, Clearfil Protect Bond, and Adper Prompt L-Pop groups than in the Clearfil SE Bond and control groups.
Conclusion: There were no statistical differences in levels of serum TNF-α and oxidative stress parameters in any groups during the course of the study. The four different adhesive systems exhibited different degrees of local toxicity to the subsurface of the skin of rats, but no systemic toxicity was detected.

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Introduction

The increasing use of esthetic restorations has led to extensive use of dental adhesives. Today, many commercial brands of bonding agents are available for clinical use. The latest generation of dentin-bonding agents seems to be simpler to use and more efficient than earlier generations.[1] However, the use of new materials with new chemical properties has raised questions concerning the biologic effects of the materials and techniques. Additionally, the reported biologic effects of dentin-bonding agents range from none to severe, depending on several factors.[2]

To evaluate the biocompatibility of dental materials, a sequence of tests must be performed, including *in vitro* assays for mutagenesis and cytotoxicity (initial tests), local toxicity reactions by intraosseous or subcutaneous implantation of the material in small laboratory animals (secondary tests), and finally usage tests.[3]

The immune system triggers inflammatory reactions to limit tissue damage against invading or foreign molecules.[4] The inflammatory response occurs in vascularized connective tissue, including plasma, circulating cells, blood vessels, and cellular and extracellular constituents of connective tissue, such as mast cells, fibroblasts and lymphocytes.[5]

Cytokines are proteins produced by many types of cells that modulate the function of other cell types. Long known to be involved in cellular immune responses, these products have additional effects that play important roles in both acute and chronic inflammation.[5] The major cytokines that mediate inflammation are interleukin 1 and tumor necrosis factor (TNF). There are two types of TNF: TNF-α and TNF-β.[6]

In the biologic evaluation of adhesive systems, one interesting possibility would be to detect the production of intracellular reactive oxidative species (ROS) induced by leachable monomers.[7] Oxidative stress (OS) is a general term used to describe the steady-state level of oxidative damage in a cell, tissue or organ caused by ROS. This damage can affect a specific molecule or the entire organism. ROS, such as free radicals and peroxides, represent a class of molecules derived from the metabolism of oxygen and inherently exist in all aerobic organisms.[8,9] Oxygen-centered free radicals are known as oxygen free radicals (OFRs).[10] Examples of OFRs are the superoxide anion ($O_2^{\bullet -}$), hydroxyl ($OH^{\bullet -}$), peroxyl ($RO_2^{\bullet -}$), alkoxyl ($RO^{\bullet -}$), and hydroperoxyl ($HO_2^{\bullet -}$) radicals.[11] These play different roles *in vivo*. However, OFRs may be very damaging, because they can oxidize lipids in cell membranes, enzymes, proteins in tissues, carbohydrates, and DNA.[9,10]

To prevent damage caused by OFRs, multiple defense systems, collectively called antioxidants, are present in serum, erythrocytes, and other organs and tissues. The antioxidant system consists of antioxidant molecules such as glutathione (GSH), vitamins A, E and C, ceruloplasmin, transferrin, albumin, and various antioxidant enzymes. Erythrocytes are excellently equipped to handle intracellular OS through the combined activity of glutathione peroxidase (GPX), and superoxide dismutase (SOD). SOD is believed to play a major role in the first line of antioxidant defense.[12]

Lipid peroxidation is the oxidative conversion of polyunsaturated fatty acid products such as malondialdehyde (MDA), which is usually measured as total thiobarbituric acid-reactive substances (TBARSs), or lipid peroxides. This is the most studied and biologically relevant free radical reaction.[13,14]

It was reported that hypertonic acidic agents applied to dentin following cavity preparation remove the smear layer and smear plugs as well as decalcify the peritubular dentin.[15] The outward dentin fluid movement then interferes with the penetration and setting of the bonding agent, from which uncured residual components that diffuse through the dentin are released during the light-curing procedure. Therefore, it is important to employ biocompatible dental materials near pulp tissues. However, few data have been published concerning tissue responses to self-etching/priming dental adhesives.[16]

Several *in vivo* studies have reported that both dental materials and their components as well as microleakage play influential roles in the inflammatory tissue response.[17,18] The aim of this study was to investigate tissue reactions of dentin bonding agents without the effect of microleakage or bacterial contamination.

The null hypotheses to be tested were: (1) there is no difference in the tissue reaction ability and local/systemic TNF-α production among the four commercially available self-etching/priming dentin-bonding agents; and (2) that all bonding agents will cause OS in rats.

Materials and methods

The study was conducted at the Ataturk University Experimental Animal and Research Center. The Ataturk University Ethics and Research Committee on the Care, Welfare and Use of Laboratory Animals approved the experimental protocol. Eighty male Sprague-Dawley rats, 80–100 days old and weighing 140–268 g, were used.

The rats were divided into 10 groups with eight rats in each, placed in cages ($60 \times 60 \times 45$ cm), and

with permitted *ad libitum* consumption of a conventional diet formulated to meet nutrient requirements assessed by the National Research Council.[19] Fresh water was also available *ad libitum* during the experiment. Rat adaptation was observed for 1 week before the experiment began.

The bonding agents used were Clearfil SE Bond (Kuraray America, Inc., New York, NY, USA), iBond (Heraeus Kulzer GmbH, Hanau, Germany), Clearfil Protect Bond (Kuraray America, Inc.), and Adper Prompt L-Pop (3M Dental Products, St. Paul, MN, USA). Applications of these agents to the rats in the groups, the durations of treatment courses, and manufacturers are shown in Tables 1 and 2. Groups CI and CII were assigned for control purposes. Group CI represented a control group of 1-week findings and Group CII of 1-month findings. Rats in the control groups were operated on, but no material was applied.

An induction mixture of ketamine hydrochloride (Ketalar, Eczacıbaşı, Lüleburgaz, Turkey) at 50 mg/kg and xylazine hydrochloride (Rompun, Bayer, Istanbul, Turkey) at 5 mg/kg was administered intramuscularly, followed by maintaining inhalation anesthesia using 1.5–4% sevoflurane (Sevorane; Abbott Laboratories, Istanbul, Turkey) volatilized with oxygen and delivered by means of a snout mask.

Table 1. Applied materials, experimental times, and groups

Group	Applied materials	Time	Weight (g)
Group SEI	Clearfil SE Bond	1 wk	190–226
Group iBI	iBond	1 wk	210–240
Group PBI	Clearfil Protect Bond	1 wk	176–242
Group PLPI	Adper Prompt L-Pop	1 wk	190–206
Group CI	Control	1 wk	166–268
Group SEII	Clearfil SE Bond	1 mo	144–190
Group iBII	iBond	1 mo	206–246
Group PBII	Clearfil Protect Bond	1 mo	174–234
Group PLPII	Adper Prompt L-Pop	1 mo	168–220
Group CII	Control	1 mo	140–208

Table 2. Components and manufacturers of the bonding agents

Classification	Product	Manufacturer	Components	Batch no.	pH
One-step self-etching	Adper Prompt L-Pop	3M Dental Products, St. Paul, MN, USA	Liquid A or compartment: methacrylated phosphoric acid esters, photoinitiator, stabilizers Liquid B or compartment: water, HEMA, polyalkenoic acid, stabilizers	187444	<1
One-step self-etching (no mixing required)	iBond	Heraeus Kulzer GmbH, Hanau, Germany	UDMA, 4-META, acetone, water, glutaraldehyde, camphorquinone	010067	2.0–2.2
Two-step self-etching	Clearfil SE Bond	Kuraray America, Inc., New York, NY, USA	Primer: MDP, HEMA, dimethacrylates, water, photoinitiator Bond: MDP, Bis-GMA, HEMA, photoinitiator, colloidal silica	389	1.9
Two-step self-etching (with an antibacterial feature)	Clearfil Protect Bond	Kuraray America, Inc., New York, NY, USA	Primer: MDP, MDPB, HEMA, water Bond: MDP, Bis-GMA, HEMA, camphorquinone, colloidal silica, NaF	Primer 5B Adeziv 10B	1.9

HEMA=hydroxyethyl methacrylate; UDMA=urethane dimethacrylate; 4-META=4-methacryloxyethyltrimellitic acid anhydride; MDP=10-methacryloyloxydecyl dihydrogen phosphate; Bis-GMA=bisphenol A diglycidyl methacrylate.

Under general anesthesia, the dorsal side was shaved and the material applied using a 10% povidone iodine antiseptic solution (Poviiodeks; Kim-Pa Co., Istanbul, Turkey), and a sterile drape was placed over the side with the animal in the lateral recumbent position. An incision was made using a 1-cm scalpel (no. 11) inserted unilaterally under the skin. A gap was prepared by inserting a retractor into the incision region to provide an application zone for the bonding agents away from the incision side. The implantation area for the applied material was then separated from the area of wound inflammation. Dental bonding systems (DBSs) were applied in accordance with the manufacturers' instructions onto subcutaneous connective tissue (Table 3). The wound was then closed and the skin sutured using 4.0 sterile sutures (Maxon 4.0, lot R77386G; Cyanamid of Great Britain, Gosport, Hampshire, UK). In the control groups, the same operation was performed but no material was applied. The animals were free to move about.

At the end of the procedure, blood from all of the rats was drawn into vacutainer tubes from the heart under the same anesthetic procedure as described above, allowed to clot, and then centrifuged at 3500*g* (5 minutes, 4ºC). The serum was immediately frozen in 1-mL aliquots and stored at −80ºC until the biochemical analyses were performed. One subject from the PLPI group died at the time of anesthesia. Specimens were thawed immediately before the assay, and hemolyzed specimens were excluded. Serum TNF-α was measured using a commercial enzyme-linked immunosorbent assay kit according to the manufacturer's instructions (series no. 11828014; Bender MedSystems, Vienna, Austria).

The subjects were sacrificed using the surgical exsanguination technique. The operative zone was extracted together with the connective tissue and fixed with 10% neutralized buffered formalin. Specimens were then embedded in paraffin, and serial sections 5 μm thick were cut on a microtome and stained with hematoxylin and eosin. All sections were blindly evaluated by two examiners for five histologic features: neutrophils, fibroblasts, lymphocytes, vascularization, and edema formation. The state of the various types of inflammatory cells, their occurrence, and tissue responses were graded from 0 to 3 as described in Table 4.

Local production of TNF-α was evaluated immunohistochemically using an anti-TNF-α kit (series no. 10026.05; DakoCytomation, Glostrup, Denmark) according to the manufacturer's protocol. Briefly, tissue samples on polylysine-coated slides were deparaffinized and rehydrated. Microwave antigen retrieval was then performed, and samples were incubated in a 3% H_2O_2 solution to inhibit endogenous peroxidase. To block nonspecific background staining, sections were incubated with a blocking solution. Sections were then incubated with a primary anti-TNF-α antibody, followed by incubation with a biotinylated goat anti-mouse antibody. After

Table 3. Application techniques of the dental bonding systems

Product	Application procedure
Adper Prompt L-Pop	1. Press compartment 1. 2. Fold the red chamber onto the yellow chamber. 3a. Press on the chambers. 3b. Spin or churn applicator to mix adhesive. 4. Apply 0.1 mL of adhesive to the connective tissue. 5. Wait 15 s. 6. Gently but thoroughly air-dry to remove the aqueous solvent. 7. Light-cure for 10 s.
iBond	1. Shake vigorously. 2. Apply 0.1 mL of adhesive to the connective tissue. 3. Wait 20 s. 4. Gently but thoroughly air-dry to remove the aqueous solvent. 5. Light-cure for 10 s.
Clearfil SE Bond	1. Apply 0.05 mL of primer to the connective tissue and let sit for 20 s. 2. Dry with mild air flow. 3. Apply 0.05 mL (total 0.1 mL) of bonding agent and distribute with gentle air flow. 4. Light-cure for 10 s.
Clearfil Protect Bond	1. Apply 0.05 mL of primer to the connective tissue and let sit for 20 s. 2. Dry gently with mild air flow. 3. Apply 0.05 mL (total 0.1 mL) of bonding agent and distribute with gentle air flow. 4. Light-cure for 10 s.

Table 4. Inflammatory tissue response

	Score 0	Score 1	Score 2	Score 3
Neutrophils	None or a few scattered neutrophils present in the operative area	Some neutrophils present in the operative area	A moderate number of neutrophils present in the operative area	Many neutrophils present in the operative area
Fibroblasts	None or a few scattered fibroblasts present in the operative area	Some fibroblasts present in the operative area	A moderate number of fibroblasts present in the operative area	Many fibroblasts present in the operative area
Lymphocytes	None or a few scattered lymphocytes present in the operative area	Some lymphocytes present in the operative area	A moderate number of lymphocytes present in the operative area	Many lymphocytes present in the operative area
Vascularization	None or slight scattered vascularization present in the operative area	Some vascularization present in the operative area	Moderate vascularization present in the operative area	Much vascularization present in the operative area
Edema	None or slight scattered edema formation present in the operative area	Some edema formation present in the operative area	Moderate edema formation present in the operative area	Severe edema formation present in the operative area

incubation with the chromogenic substrate (DAB), sections were counterstained with hematoxylin and eosin.

The intensities of local TNF-α levels and tissue reactions were evaluated using a light microscope (100× and 200× magnification; Olympus BX51; Olympus Europa Holding, Hamburg, Germany). All analyses were performed by two pathologists who were blinded to the group assignments. The evaluation of staining of cytoplasmic TNF-α in the tissue was scored as a percentage of results, and the tissue reactions were classified as mild, moderate or severe.

To measure OS, 1 mL of blood was taken from the heart using a 24-gauge angiocatheter (Hayat Medical Instruments Co., Istanbul, Turkey) 1 week and 1 month after implantation. Erythrocyte sediments were prepared for the analyses. Erythrocytes were then hemolyzed by diluting with deionized water (50-fold), and the analyses were carried out in these hemolyzed supernatant fractions. Hemoglobin (Hb) values of samples were measured using a Gen-S counter hematology analyzer (Beckman Coulter, Inc., Fullerton, CA, USA). Hemolysate samples were kept at −80ºC until biochemical determination.

The MDA measurement, an important indicator of OS, was based on the spectrophotometric absorbance of the pink-colored product of the thiobarbituric acid-reactive substance (TBARS) complex.[20] Total TBARSs were expressed as MDA. Results are expressed as nanomole per gram of Hb. SOD activity was measured by nitroblue tetrazolium reduction by $O_2^{\bullet -}$ generated by the xanthine/xanthine oxidase system.[21] SOD activity was measured at 560 nm by detecting the inhibition of this reaction, and was expressed as unit per milligram of Hb. GPX activity was detected according to the method described by Paglia and Valentine.[22] By measuring the absorbance change in NADPH at 340 nm per minute and using the molar extinction coefficient of NADPH, GPX activity was calculated as unit per gram of Hb. The total GSH level was measured spectrophotometrically at 412 nm using a glutathione disulfide reductase recycling method, as described by Tietze.[23] In this method, the rate of yellow-colored 5-thio-2-nitrobenzoic acid production is directly proportional to the concentration of GSH in the sample. Results were expressed as micromole per gram of Hb.

Statistical analyses were carried out using analysis of variance among MDA, GSH, SOD, and GPX levels, and serum and tissue TNF-α levels. Differences between groups were evaluated using Duncan's multiple comparison test at a significance level of $P<0.05$.

For the statistical analysis of the tissue reaction, Mann-Whitney *U* and Kruskal-Wallis tests were performed to determine whether there was a statistically significant difference ($P<0.05$) among ranked groups and times.

Results

Gross findings

In the 1-week findings, Groups CI and SEI, with the exception of two subjects (for which exudate was observed after scab removal), showed healing in the wound. However, there was no evidence of healing in the operative zone, and a thick scab,

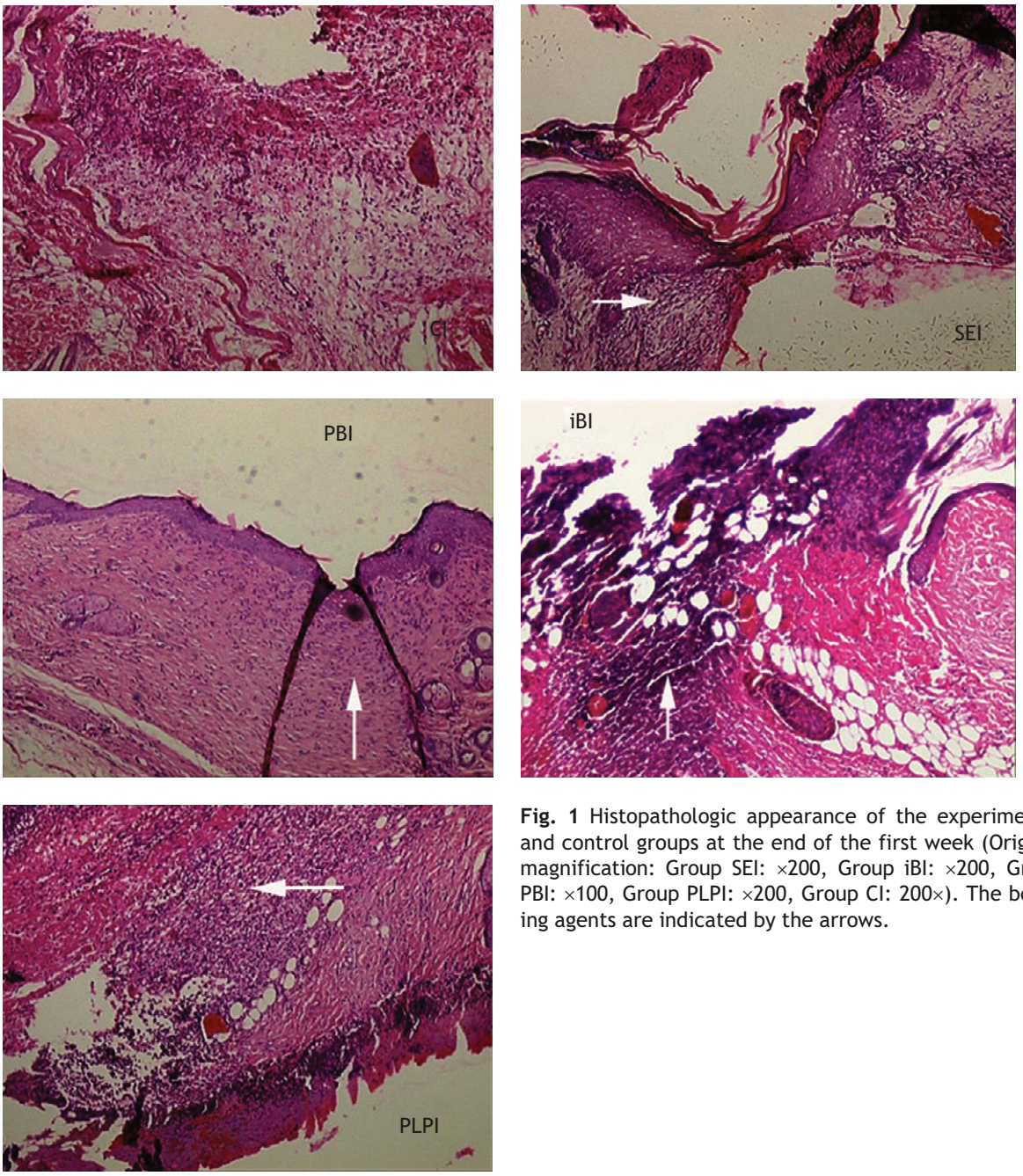

Fig. 1 Histopathologic appearance of the experimental and control groups at the end of the first week (Original magnification: Group SEI: ×200, Group iBI: ×200, Group PBI: ×100, Group PLPI: ×200, Group CI: 200×). The bonding agents are indicated by the arrows.

inflammation and exudate were present in all subjects in the groups.

In the 1-month findings, the SEII, PBII and CII groups exhibited healed wounds, and no scabs were seen. However, no healing was observed in two subjects in the iBII group and one in the PLPII group.

Histopathologic findings at 1 week

Fibroblast levels in Group SEI were higher than in the other groups ($P<0.01$). Although neutrophil and vascularization levels were similar to those in the control group, edema formation and lymphocyte counts were slightly higher than in Group CI (control group, 1-week findings), although the difference was not statistically significant (Fig. 1).

Neutrophil, vascularization and edema levels were significantly higher in Group iBI compared with the other groups ($P<0.01$), and fibroblast levels were lower than those in the other groups ($P<0.01$), except for the controls (Fig. 1).

Vascularization and edema formation were higher in the PBI group than in the SEI and CI groups. Neutrophil levels were higher in the PBI group than in all other groups ($P<0.01$) except for Group iBI at 1 week (Fig. 1).

Neutrophil levels were higher in Group PLPI than in Groups SEI and CI ($P<0.01$) but were lower

than in Group iBI. Lymphocyte and fibroblast levels were higher than in the other groups, except for Group SEI. However, these findings were not statistically significant. In addition, the vascularization level was lower in Group PLPI than in Groups iBI and PBI and higher than in the controls and in Group SEI ($P<0.01$) (Fig. 1).

Levels of lymphocyte and neutrophil infiltration were low in density in Group CI (control). Edema and fibroblast levels were determined to be small, except in two subjects. The neutrophil level in the control group differed from that in Groups iBI and PBI ($P<0.01$). Fibroblast and vascularization levels differed from those in Group iBI ($P<0.01$) (Fig. 1).

Histopathologic findings at 1 month

Fibroblast and lymphocyte counts were higher in Group SEII than in Group CII (1-month findings in the control group; $P<0.001$ for fibroblasts and $P<0.01$ for lymphocytes), but the other data were similar to the controls. When compared with the 1-week findings, neutrophil and vascularization levels had decreased ($P<0.05$) but the fibroblast level had increased ($P<0.05$; Fig. 2).

Although neutrophil and lymphocyte levels were higher in Group iBII than in the other groups ($P<0.05$), the fibroblast level was lower ($P<0.001$). Compared with the 1-week findings, the fibroblast

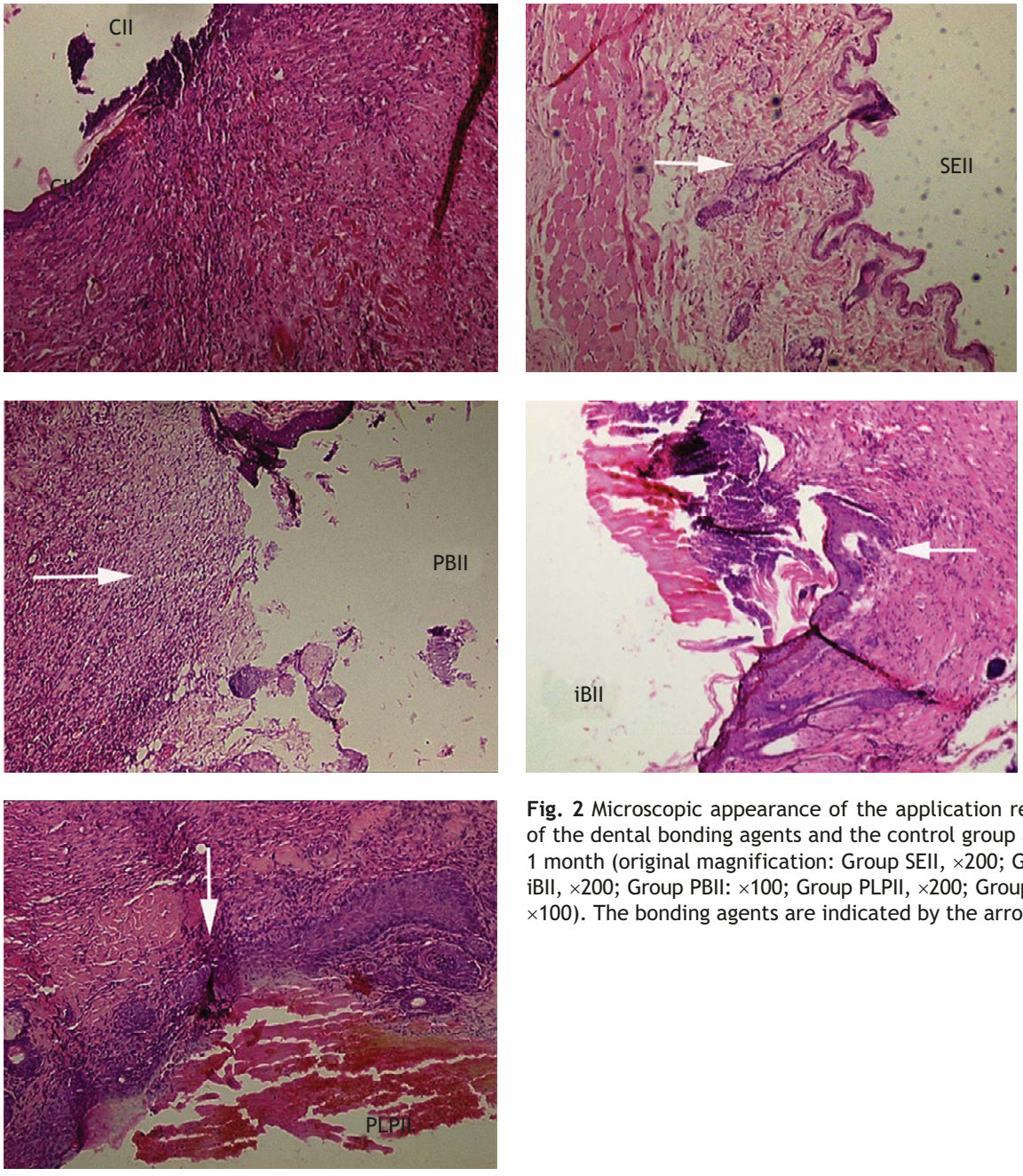

Fig. 2 Microscopic appearance of the application region of the dental bonding agents and the control group after 1 month (original magnification: Group SEII, ×200; Group iBII, ×200; Group PBII: ×100; Group PLPII, ×200; Group CII, ×100). The bonding agents are indicated by the arrows.

level had increased ($P<0.05$) but edema, neutrophil ($P<0.05$), and vascularization ($P<0.01$) levels had decreased (Fig. 2).

All findings in Group PBII were higher than those in Group CII. When compared with the 1-week data, fibroblastic activity had risen ($P<0.05$) but neutrophil ($P<0.05$) and vascularization levels ($P<0.01$) had decreased (Fig. 2).

All findings in Group PLPII were higher than in the controls. Lymphocyte activity differed from those in groups SEII and PBII. Compared with 1-week data, fibroblastic activity had risen ($P<0.05$) but the other findings had decreased ($P<0.05$) (Fig. 2).

All inflammation values in Group CII (control) were lower than those in the other 1-month groups. The values were lower in degree, and all findings were statistically significant ($P<0.05$), except for the vascularization level compared with group SEII (Fig. 2). The statistical analyses showed that the 1-week and 1-month findings differed with respect to neutrophil ($P<0.05$), fibroblast ($P<0.05$) and vascularization levels ($P<0.01$).

Tissue TNF-α

During the first week, the result for local TNF-α secretion in Group CI (control) was statistically lower than in the other groups. In Groups SEI and PBI, TNF-α levels were higher than in the controls but lower than in Groups iBI and PLPI. The TNF-α receptor-binding level was concentrated in Groups iBI and PLPI. Differences between Groups SEI and PBI and between Groups iBI and PLPI were not significant, but those between the first-week groups and Group CI were significant ($P<0.001$) (Fig. 3).

At the end of the first month, no differences were determined between Groups SEII and CII (control) in terms of TNF-α levels, but Groups iBII, PBII and PLPII statistically differed from Groups CII and SEII. Differences in Groups iBII, PBII and PLPII were statistically significant ($P<0.001$; Fig. 4).

In addition, the TNF-α level in the control group decreased compared with the first-week level. In Groups iBII and PBII, TNF-α values were lower compared with those in the first week, but were still higher than those in Groups SEII and PLPII ($P<0.001$; Table 5).

Biochemical findings

There were no differences between the control and the other groups in terms of serum TNF-α and OFR production (MDA, GSH, SOD, and GPX) levels at either 1 week or 1 month. Results of the study are summarized in Tables 5–7.

Discussion

No studies have so far investigated the biocompatibility of the systemic toxicity of DBSs via ROS production. We determined no differences between the control and experimental groups in terms of MDA, GSH, SOD, and GPX values, important indicators of OS.

The biocompatibility of dentin-bonding agents is imperative, since they are placed on etched dentin near the pulp, where tubular density and diameter are the greatest.[24]

With all materials used in restorative dentistry, there is some risk of biologic reactions because of incomplete polymerization. DBSs are usually polymerized by photoactivation, and free monomers may be released from resinous materials before and after polymerization. Theoretically, a 100% conversion of monomers to polymers is possible, but as much as 25–50% of the methacrylate monomer double-bonds actually remain unreacted in the polymer.[25] The unpolymerized monomer may be responsible for biologic reactions if it passes through the dentinal tubules and reaches the pulp tissue.[26]

The immune system triggers inflammatory reactions to limit tissue damage from invading or foreign molecules.[4] In considering compatibility and its relationship to other *in vivo* elements following implantation tests, appreciation of wound healing is essential.[27] The first phase of healing is the acute inflammatory response, which includes exudation of fluid and plasma proteins (edema) and the emigration of leukocytes, predominantly neutrophils. Following the acute inflammatory response, chronic inflammation and normal wound healing occur, with the presence of lymphocytes and macrophages, the proliferation of blood vessels, and fibroblasts.[5] We used edema, and neutrophil and lymphocyte levels to assess the acute inflammatory tissue response and fibroblast and vascularization levels to assess wound healing levels. We found that when adhesive systems were applied to connective tissue, they caused an inflammatory tissue response and delayed the wound healing time compared with the control groups, at both 1 week and 1 month.

Some researchers recently determined that no adverse effect occurs when adhesive systems are applied as pulp capping materials, in spite of the manufacturers' instructions to the contrary.[28] Consequently, the biocompatibility and cytotoxicity of dental composites and their components have been analyzed, because these materials were initially recommended for application to dentin or, more recently, for direct pulp capping.[29] Jontell et al.[30] emphasized that resin components may evoke an immune reaction by spleen cells. In our study, all DBSs exhibited inflammatory reactions to differing

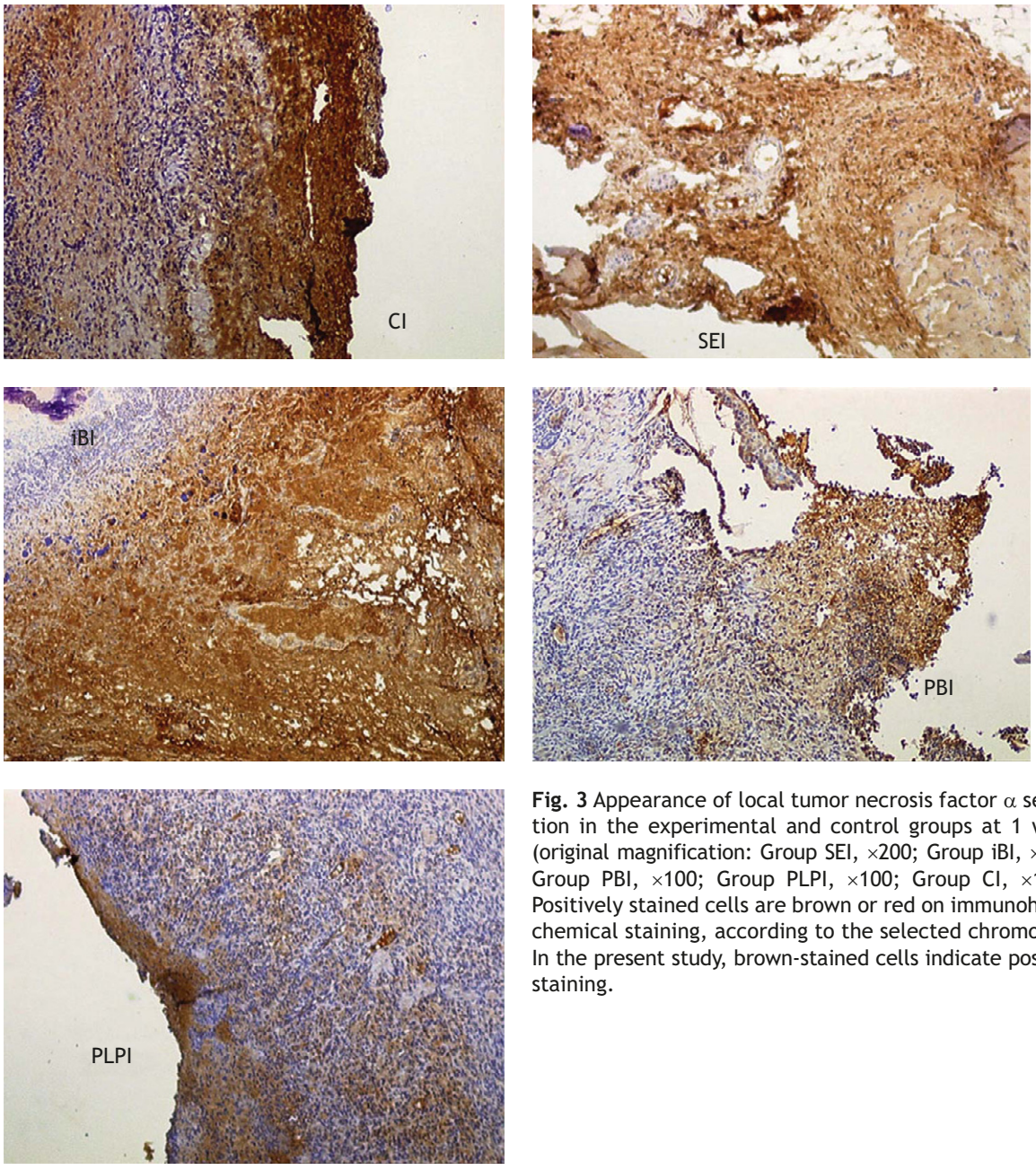

Fig. 3 Appearance of local tumor necrosis factor α secretion in the experimental and control groups at 1 week (original magnification: Group SEI, ×200; Group iBI, ×100; Group PBI, ×100; Group PLPI, ×100; Group CI, ×100). Positively stained cells are brown or red on immunohistochemical staining, according to the selected chromogen. In the present study, brown-stained cells indicate positive staining.

degrees. Wound healing was delayed in all subjects compared with control groups at both 1 week and 1 month. Reactions induced by DBSs can also enhance their acidity.[31] We used four different self-etching/priming DBSs with different acidities. Some researchers suggested that applying self-etching/priming adhesive systems to contacted pulp of healthy dog teeth does not lead to acceptable repair of the dentine-pulp complex.[32] Additionally, de Souza Costa et al.[33] determined that calcium hydroxide remains the pulp capping agent of choice for mechanically exposed human pulp. Self-etching adhesive systems do not allow complete connective tissue repair adjacent to the pulp exposure site. These findings are in agreement with those of the present study.

TNF-α is an inflammatory cytokine produced by macrophages/monocytes during acute inflammation, is responsible for a diverse range of signaling events within cells, and leads to necrosis or apoptosis. Proteins are also important for resistance to infection.[6]

We used local TNF-α levels to compare the biologic reactions of the DBSs. The eight working groups exhibited differing degrees of tissue reactions. Inflammation was maintained at different levels at 1 week and 1 month. In addition, wound healing was slow in all groups compared with the

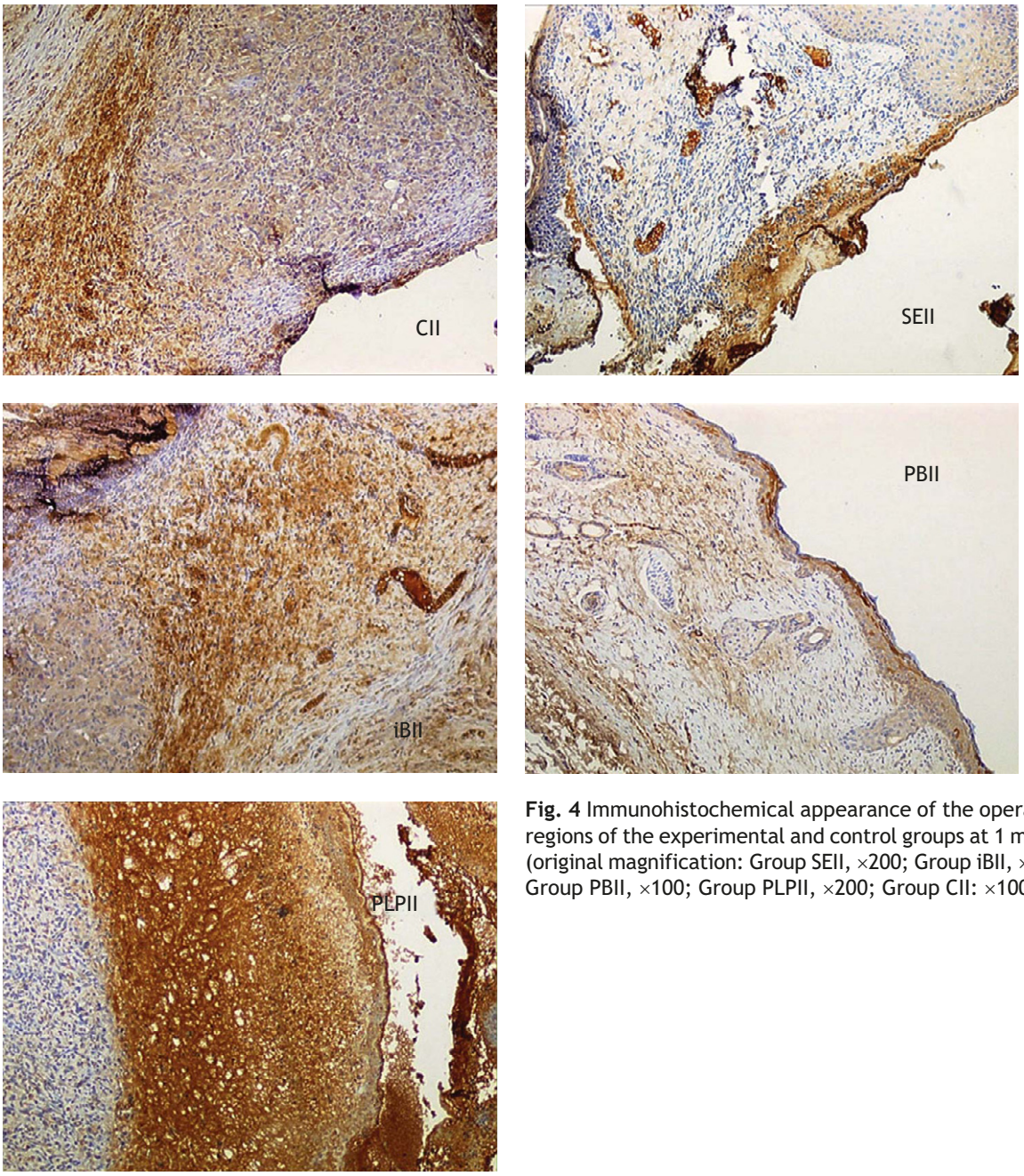

Fig. 4 Immunohistochemical appearance of the operative regions of the experimental and control groups at 1 month (original magnification: Group SEII, ×200; Group iBII, ×100; Group PBII, ×100; Group PLPII, ×200; Group CII: ×100).

Table 5. Tissue tumor necrosis factor (TNF) α level*

	Control	Clearfil SE Bond	iBond	Clearfil Protect Bond	Adper Prompt L-Pop
TNF-α, mean (SD) (%)					
1 wk	23.125[†] (9.6130)	38.75[‡] (19.5941)	78.7500[§] (11.2599)	47.50[‡] (7.5592)	76.42[§] (14.6385)
1 mo	13.750[‖] (4.4320)	15.00[‖] (7.0710)	70.62[¶] (10.5008)	25.00[#] (10.3509)	16.87[‖#] (5.9386)

*Means with different symbols in a given row statistically differ ($P<0.05$). SD=standard deviation.

Table 6. Neutrophil, fibroblast, vascularization, and edema scores at 1 week and 1 month

	Control	Clearfil SE Bond	iBond	Clearfil Protect Bond	Adper Prompt L-Pop
Neutrophil score (%)					
1 wk					
1	87.5	62.5	–	12.5	42.9
2	12.5	25.0	37.5	62.5	42.9
3	–	12.5	62.5	25.0	14.2
1 mo					
1	87.5	75.0	25.0	75.0	50.0
2	12.5	25.0	50.0	25.0	50.0
3	–	–	25.0	–	–
Fibroblast score (%)					
1 wk					
1	87.5	12.5	75.0	50.0	42.9
2	12.5	25.0	25.0	50.0	42.9
3	–	62.5	–	–	14.2
1 mo					
1	87.5	–	50.0	37.5	87.5
2	12.5	37.5	50.0	62.5	12.5
3	–	62.5	–	–	–
Lymphocyte score (%)					
1 wk					
1	100	37.5	62.5	50.0	42.9
2	–	50.0	37.5	50.0	42.9
3	–	12.5	–	–	14.2
1 mo					
1	75.0	12.5	–	50.0	62.5
2	25.0	62.5	75.0	50.0	37.5
3	–	25.0	25.0	–	–
Vascularization score (%)					
1 wk					
1	62.5	50.0	–	25.0	57.2
2	37.5	50.0	37.5	62.5	28.6
3	–	–	62.5	12.5	14.2
1 mo					
1	87.5	87.5	75.0	62.5	50.0
2	12.5	12.5	25.0	37.5	50.0
3	–	–	–	–	–
Edema score (%)					
1 wk					
1	87.5	37.5	–	37.5	28.6
2	12.5	50.0	37.5	50.0	42.9
3	–	12.5	62.5	12.5	28.6
1 mo					
1	75.0	75.0	25.0	37.5	62.5
2	25.0	25.0	50.0	50.0	37.5
3	–	–	25.0	12.5	–

control groups in terms of histopathologic and biochemical findings. Our results are in agreement with those of Cortes et al.[34] who determined localized abscess formation and no dentinal bridge formation after applying different adhesives to rat molar tooth pulp. Rakich et al.[35] also demonstrated that the application of dentin bonding agents to cell culture medium causes secretion of TNF-α from macrophages. However, other researchers reported that resin-based materials allow pulp healing and tertiary dentine deposition.[28]

In this study, tissue levels of TNF-α were compared to evaluate systemic biologic reactions to the various dentin bonding agents. No systemic toxicity derived from the dentin bonding agents was determined. The findings of this study are compatible

Table 7. Serum tumor necrosis factor (TNF) α, malondialdehyde (MDA), and glutathione (GSH) levels and superoxide dismutase (SOD) and glutathione peroxidase (GPX) activities*

	Control	Clearfil SE Bond	iBond	Clearfil Protect Bond	Adper Prompt L-Pop
MDA (nmol/g Hb)					
1 wk	5.52 (1.09)	4.66 (2.15)	5.98 (1.98)	4.92 (2.06)	4.05 (1.78)
1 mo	4.67 (0.19)	6.58 (2.42)	3.8 (1.69)	5.12 (0.84)	5.94 (2.54)
SOD (U/mg Hb)					
1 wk	226.23 (132.31)	240.4 (146.5)	249.79 (154.49)	176.85 (141.85)	248.38 (190.63)
1 mo	294.44 (46.46)	273.59 (177.99)	327.15 (175.84)	374.04 (173.94)	248.47 (81.29)
GSH (μmol/g Hb)					
1 wk	53.64 (17.27)	75.96 (44.69)	113.96 (48.64)	72.06 (40.82)	88.07 (45.09)
1 mo	81.41 (19.33)	76.55 (45.09)	50.73 (27.12)	98.22 (48.54)	83.54 (23.6)
GPX (U/g Hb)					
1 wk	9.66 (4.89)	8.11 (3.23)	7.95 (3.22)	8.06 (4.57)	9.86 (5.194)
1 mo	8.54 (4.79)	8.42 (2.49)	9.2 (2.3)	11.79 (6.17)	9.6 (4.4)
TNF-α (pg/mL)					
1 wk	$437.25^{\dagger}$ (73.43)	$630.63^{\dagger\ddagger}$ (103.39)	$774.38^{\ddagger}$ (94.97)	$484.38^{\dagger\ddagger}$ (94.21)	$702.86^{\dagger\ddagger}$ (147.59)
1 mo	$366.875^{\S}$ (35.41)	$615.63^{\|}$ (54.11)	$362.5^{\S}$ (89.36)	$438.13^{\S\|}$ (88.21)	$554.38^{\S\|}$ (94.02)

*Values are expressed as mean (standard deviation), and means with different symbols in a given row significantly differ ($P<0.05$). Hb=hemoglobin.

with previous results that dentin bonding agents cause no systemic toxicity, because resin-based materials release components in relatively small amounts.[36] Therefore, systemic toxicity is of less value for assessing the biocompatibility of resin-based dental materials.

OFRs may directly induce cell damage, or act as an intracellular messenger during cell death induced by various other kinds of stimuli.[37] Recently, OFR production was described as an early expression of cellular stress in dental monomer cytotoxicity. Some components of resin-based dental materials, such as monomers and photoinitiators, were described as increasing OFR production.[38–40] Furthermore, assaying enzymatic and non-enzymatic antioxidants provides an indirect assessment of OFR generation in OS.[41] When the balance between OFR production and antioxidative defense mechanisms is impaired, OFR levels may rise. When the OFRs are not removed by natural scavengers, damage occurs through peroxidation within the phospholipid structure of membranes.[42]

In conclusion, the null hypotheses were rejected. Histopathologic findings in this study demonstrated that DBSs caused local toxicity and delayed wound healing to different degrees when applied to connective tissue of rats. However, the findings also showed that DBSs did not induce oxidative stress or an increase in serum TNF-α. Further studies are needed to evaluate the biocompatibility of components of such adhesives and to determine the possible causes of these tissue reactions.

Acknowledgments

This study was supported by a grant-in-aid (2003-166) from the Ataturk University Scientific Project Research Foundation, Turkey. The iBond and Clearfil Protect Bond used in this study was generously supplied by Heraeus Kulzer GmbH and the Dental Department of Kuraray America, Inc. We thank Dr Yunus Emre Ozkanlar for critically reviewing the manuscript.

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